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Award Number: DAMD17-03-1-0120

TITLE: The Role of SDF-1 α /CXCR4/MMP in PC Bone Metastasis

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REPORT DATE: March 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

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| 1. AGENCY USE ONLY (Leave blank) | | 2. REPORT DATE March 2005 | 3. REPORT TYPE AND DATES COVERED Annual (1 Mar 2004 - 28 Feb 2005) |
| 4. TITLE AND SUBTITLE The Role of SDF-1 α /CXCR4/MMP in PC Bone Metastasis | | | 5. FUNDING NUMBERS DAMD17-03-1-0120 |
| 6. AUTHOR(S) Sreenivasa R. Chinni, Ph.D. | | | |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Wayne State University Detroit, Michigan 48201-1969 E-Mail: schinni@med.wayne.edu | | | 8. PERFORMING ORGANIZATION REPORT NUMBER |
| 9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012 | | | 10. SPONSORING / MONITORING AGENCY REPORT NUMBER |
| 11. SUPPLEMENTARY NOTES | | | |
| 12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited | | | 12b. DISTRIBUTION CODE |
| 13. ABSTRACT (Maximum 200 Words) Chemokines and chemokine receptor interactions facilitate the physiological migration of cells. Interaction of chemokines with their receptors leads to the expression/activation of adhesion molecules and proteases. Recent evidence suggests that a similar mechanism may be active in cancer metastasis. Herein, we hypothesize that CXCL12 and CXCR4 interactions facilitates the metastasis of prostate cancer cells by activating intracellular signaling pathways leading to the expression and release of MMP-9. Using a variety of methods including RT-PCR, ELISA, gelatin zymography, cellular motility and invasion and subcellular fractionation of prostate cancer cells, we showed that (a) bone stromal cells and bone tissue conditioned media induced the migration of PC-3 cells in a CXCR4 dependent manner; (b) pharmacological inhibition of PI3 kinase and MAP kinase pathways abrogated the CXCL12 induced invasion of PC-3 cells; (c) CXCL12 induced Akt1 phosphorylation and Akt1 siRNA transfections abrogated the CXCL12 induced Akt phosphorylation, proMMP-9 secretion, migration and invasion of PC-3 cells. This data suggests that chemoattractive mechanisms may involve migration of cancer cells towards bone tissue, and that cell signaling induced by binding of the chemokine to its receptor leads to the activation of multiple signaling pathways and subsequent release of MMPs into the local environment. | | | |
| 14. SUBJECT TERMS CXCR4, SDF-1 α , MMP, prostate cancer bone metastasis | | | 15. NUMBER OF PAGES 10 |
| | | | 16. PRICE CODE |
| 17. SECURITY CLASSIFICATION OF REPORT Unclassified | 18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified | 19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified | 20. LIMITATION OF ABSTRACT Unlimited |

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INTRODUCTION:

Prostate cancer cells frequently metastasize to bone. The bone-associated chemokines may play a role in the chemoattraction of prostate cancer cells to bone. The interaction of bone associated chemokines with the chemokine receptors on prostate cancer cells leads to the activation of signaling pathways and subsequent expression of proteases including MMP-9. The literature suggests the chemokine, CXCL12 formerly known as SDF-1 α , and its cognate receptor CXCR4 are involved in the metastasis of breast cancer cell to lymph node and lung(1), further several recent studies show the existence these chemokine and chemokine receptor interactions in several types of cancers including, prostate(2, 3), ovarian(4), melanoma(5), colon(6), pancreatic(7, 8) and glioblastoma (3, 9) tumors. The purpose of current proposal is to demonstrate the presence of functional chemokine receptor; CXCR4 in PC cells and clinical PC bone metastasis and to test the hypothesis that interaction of bone associated CXCL12 with CXCR4 expressed on prostate cancer cells elicits intra cellular signaling events leading to the MMP-9 gene expression. The experiments in the current proposal were designed to provide link between the chemoattraction of cancer towards target environment mediated by the CXCL12 and CXCR4, and expression of proteases by activation of cell signaling pathways in the bone target environment.

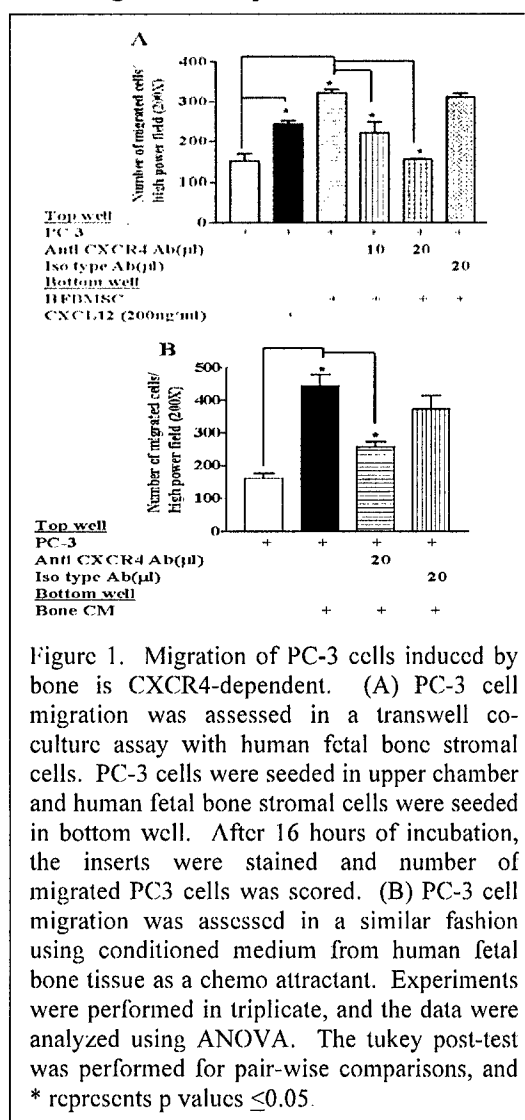
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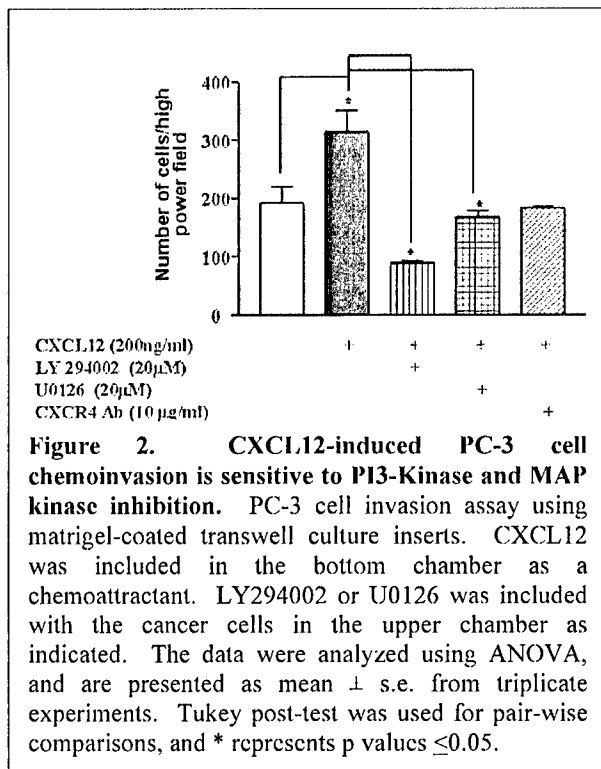
Bone cell-associated CXCL12 stimulates CXCR4-dependent migration of prostate cancer cells.

To study the role of stromal cell-associated soluble CXCL12 in inducing chemomigration of PC-3 cells, we employed a transwell co-culture system where prostate cancer cells were cultured with either bone stromal cells or bone tissue conditioned medium. We showed that purified CXCL12, bone stromal cells, and bone tissue conditioned medium all stimulated chemomigration of PC-3 cells. Pretreatment with anti-CXCR4 antibody inhibited PC-3 cell migration towards stromal cells in a dose dependent fashion (Figure 1A). Pre-treatment of PC-3 cells with anti-CXCR4 antibody similarly inhibited bone conditioned media-induced chemomigration (Figure 1B). These data indicated that cancer cell migration in bone co-cultures is dependent on CXCL12/CXCR4 signaling.

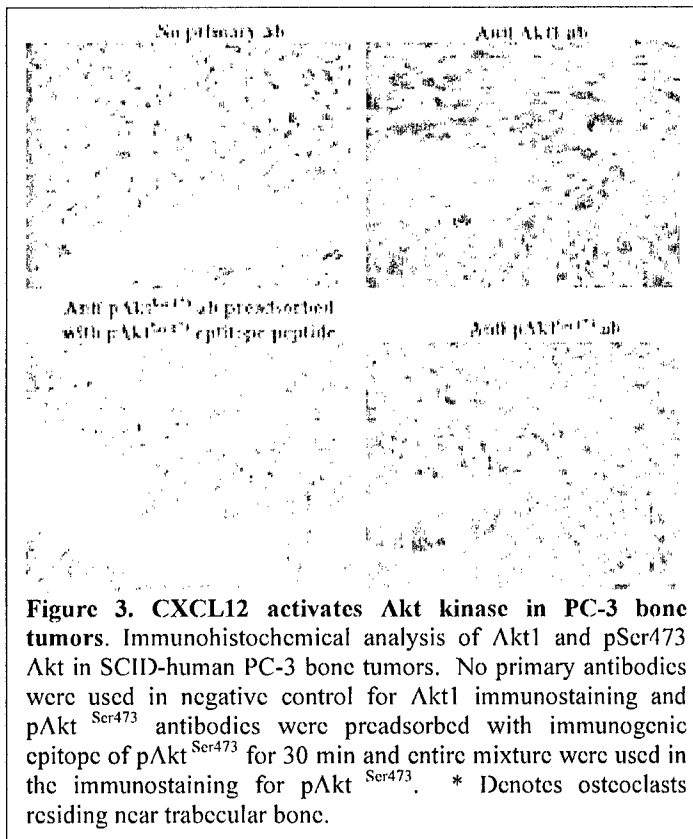
CXCL12-mediated PC-3 cell chemoinvasion is sensitive to PI3-Kinase and MEK kinase inhibitors.

The PI3 kinase and MAP kinase pathways have been shown to be activated by CXCL12/CXCR4 interaction (2, 10, 11). Pretreatment of PC-3 cells with either LY294002 (PI3 kinase inhibitor) or U0126 (MEK inhibitor) abrogated CXCL12-induced MMP-9 gene expression (Annual Report 04). One of the biological activities of cancer cell-derived MMPs are to facilitate invasion of the extracellular matrix (12, 13). Using matrigel coated cell culture inserts, we showed that pharmacological inhibition of either the PI3 kinase or MAP kinase pathway abolished CXCL12-induced chemoinvasion of PC-3 cells (Figure 2). LY294002 appeared to be more potent than U0126 in inhibiting CXCL12-induced MMP-9 gene (Annual Report 04) and



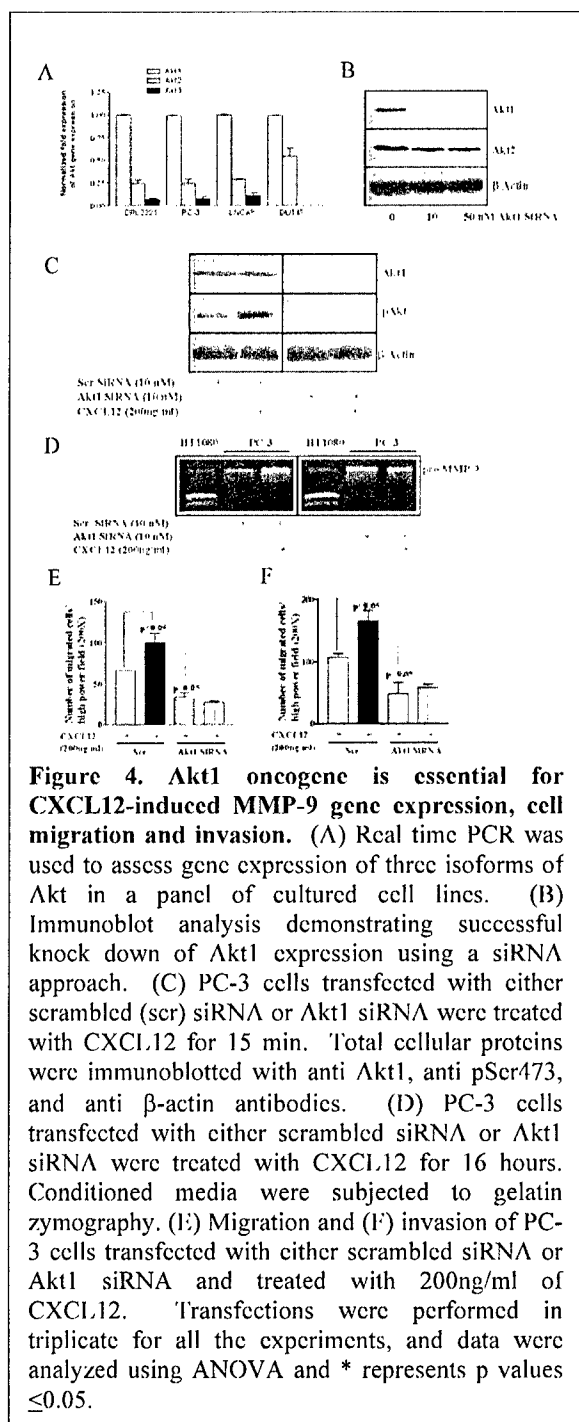


protein expression as well as chemoinvasion in prostate cancer cells.



CXCL12-induced Akt phosphorylation in PC-3 cells. Published results show that CXCL12 activates Erk 1 and 2 (2), as well as Akt (11) in different prostate cancer cell lines (11). Here, we found that CXCL12 stimulation led to activation of Akt in PC-3 cells (Annual Report 04), and this activation was sensitive to pretreatment of PC-3 cells with LY294002. These data suggested that Akt phosphorylation was mediated via the PI3 kinase pathway. Immunohistochemical analysis of SCID-human PC-3 bone tumors demonstrated specific staining for both Akt1 and pAkt^{Ser473} in both cancer cells and multinucleated osteoclasts residing near bone trabeculae (Figure 3).

Akt1 kinase activation is indispensable for CXCL12 induced MMP-9 gene expression, release, migration and invasion of PC-3 cells. Previous studies showed that the three known isoforms of Akt were expressed in prostate cancer cells (14, 15). We used a sensitive real time PCR method to quantify the relative abundance of the various Akt isoforms in our cells (Figure 4 A). We found that the Akt1 gene is the most abundant isoform expressed in all the cell lines tested. To further understand the role CXCL12-induced activation of the PI3 kinase pathway in proMMP-9 secretion, cell migration, and invasion, we employed siRNA methodology to knock down Akt1. Western blot analysis of cells transiently transfected with Akt1 siRNA showed that Akt 1 protein expression was specifically down regulated in PC-3 cells (Figure 4B). Furthermore, down regulation of the Akt1 isoform by siRNA diminished the overall levels of 473pSer-activated Akt (Figure 4C), suggesting that CXCL12 signaling involves the Akt1 isoform in PC-3 cells. We then stimulated the Akt1 siRNA-transfected cells and control-transfected cell with CXCL12 and found that MMP-9 secretion was inhibited only in the Akt1 si-RNA transfected cells (Figure 4D). Furthermore, Akt1 siRNA transfection specifically inhibited CXCL12-induced migration (Figure 4E) and invasion (Figure 4F). Together, these data showed that Akt1 signaling was unequivocally involved in CXCL12-induced MMP-9 secretion, migration, and invasion by PC-3 cells.

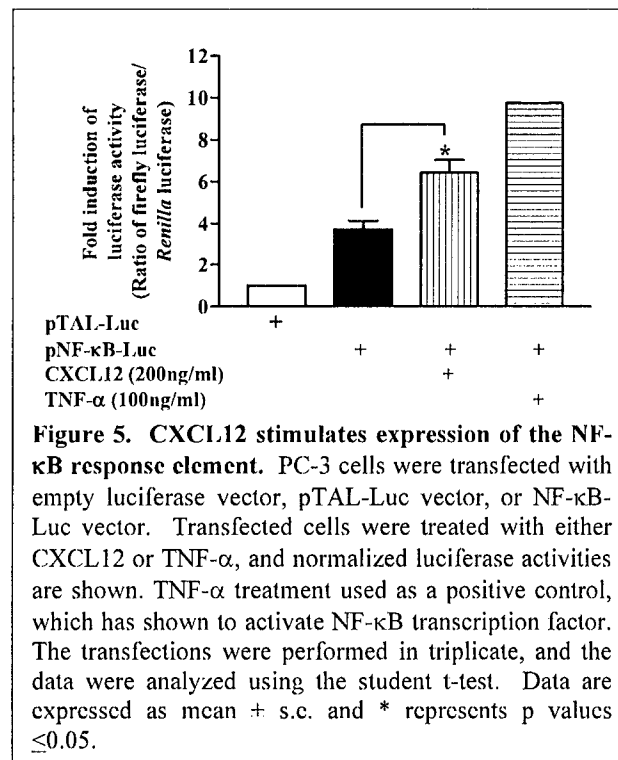


suggest that CXCL12/CXCR4-mediated PI3K/Akt activation leads to expression of NF- κ B responsive genes.

KEY RESEARCH ACCOMPLISHMENTS

- Bone tissue associated CXCL12 stimulates CXCR4 dependent PC-3 cell chemomigration.
- CXCL12 mediated chemoinvasion of PC-3 cells is sensitive to both PI3 kinase and MAP kinase inhibitors.
- SCID-human PC-3 bone tumors express Akt protein and activated Akt protein. Tumor cells and osteoclasts show immunoreactivity for both Akt and phosphorylated Akt protein.
- Akt 1 phosphorylation is indispensable for CXCL12 induced MMP-9 gene expression, secretion, migration and invasion of PC-3 cells.
- CXCL12/CXCR4 interactions activates NF- κ B response element in PC-3 cells.

CXCL12/CXCR4 interaction leads to activation of the NF- κ B response element. Recent studies showed that activated Akt transduces signals via the cellular activation of NF- κ B transcription factor (16, 17). Among the secreted MMPs, only MMP-9 has an NF- κ B transcription factor binding element in the promoter sequence (18), and we previously showed that PC-3 cells are capable of responding to external stimuli by activating NF- κ B transcription factor activity (19). Here we evaluated whether CXCL12/CXCR4 signaling can act via NF- κ B. We transfected PC-3 cells with an NF- κ B response



element fused to a luciferase reporter construct. CXCL12 treatment of the transfected cells led to luciferase expression demonstrating activation of the NF- κ B response element (Figure 5). These data

REPORTABLE OUTCOMES

Abstract: **Chinni, SR;** Sivalogan S; Dong, Z; Trindade Filho, JC; Deng, X; Bonfil RD and Cher, ML. CXCL12/CXCR4 signaling induces akt activation and mmp-9 expression in prostate cancer cells. Eleventh Annual Scientific Retreat of Prostate Cancer Foundation, Lake Tahoe, Nevada, October 21-24, 2004.

CONCLUSIONS

These results suggest that chemokine, CXCL12 and its cognate receptor CXCR4 are expressed in the cellular phenotypes of prostate cancer bone metastasis. Bone associated CXCL12 could act as a chemoattractant for circulating Prostate Cancer cells. Biological activities of prostate cancer cell associated CXCL12/CXCR4 interactions include expression and secretion of MMP-9. CXCL12 induced cell signaling pathways mediate chemoinvasion of cancer cells, and activated Akt 1 kinase is indispensable for CXCL12 mediated cellular migration and invasion of prostate cancer cells. This data suggests that chemoattractive mechanisms may involve migration of cancer cells towards bone tissue, and that cell signaling induced by binding of the chemokine to its receptor leads to the activation of multiple signaling pathways and subsequent release of MMPs into the local environment. These findings may provide a link between chemoattractive mechanisms, growth of tumor cells in bone, and tumor-enhanced bone matrix turnover.

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APPENDICES

Appendix 1

CXCL12/CXCR4 SIGNALING INDUCES AKT ACTIVATION AND MMP-9 EXPRESSION IN PROSTATE CANCER CELLS

Sreenivasa R. Chinni, Sivasakthy Sivalogan, Zhong Dong, J. Carlos Trindade Filho, R. Daniel Bonfil and Michael L. Cher.

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Chemokines and chemokine receptor interactions facilitate the physiological migration of cells. Hematopoietic cells home to bone by means of chemo-attraction to marrow chemokines. Interaction of chemokines with their receptors leads to the expression/activation of adhesion molecules and proteases. Recent evidence suggests that a similar mechanism may be active in cancer metastasis: expression of the chemokine CXCL12 and its receptor CXCR4 has been documented in several epithelial tumors. We previously showed that broad-spectrum inhibition of matrix metalloprotease (MMP) activity diminishes bone matrix turnover and proliferation of prostate cancer (PC) cells in bone. Using specific tissue-based enzymatic activity assays, we recently demonstrated an increase in net MMP-9 activity, but not MMP-2 or MT1-MMP activity, in bone tissues colonized by cancer cells *in vivo*. The MMP-9 protein immunolocalized to prostate cancer cells and osteoclasts. Herein, we hypothesize that CXCL12 and CXCR4 interactions facilitates the metastasis of prostate cancer cells by activating intracellular signaling pathways leading to the expression and release of MMP-9. Using a variety of methods including RT-PCR, ELISA, gelatin zymography, cellular motility and invasion and subcellular fractionation of prostate cancer cells, we showed that (a) CXCR4 was expressed on prostate cancer cells and experimental *scid*-human PC-3 bone tumors and CXCL12 was expressed on stromal cells of bone and lung and to a lesser extent in PC-3 cells; (b) CXCL12 induced MMP-9 gene expression and secretion in prostate cancer cells; (c) bone stromal cells and bone tissue conditioned media induced the migration of PC-3 cells in a CXCR4 dependent manner; (d) pharmacological inhibition of PI3 kinase and MAP kinase pathways abrogated the CXCL12 induced MMP-9 gene expression and release, and invasion of PC-3 cells; (e) CXCL12 induced Akt1 phosphorylation and Akt1 siRNA transfections abrogated the CXCL12 induced Akt phosphorylation, proMMP-9 secretion, migration and invasion of PC-3 cells; (f) CXCR4 was localized to lipid rafts in prostate cancer cells and CXCL12 induced HIF1 phosphorylation in PC-3 cells; (g) kinetics of CXCL12 induced HIF1 and Akt phosphorylation showed that CXCL12 induced HIF1 phosphorylation preceded Akt phosphorylation in PC-3 cells. This data suggests that chemoattractive mechanisms may involve migration of cancer cells towards bone tissue, and that cell signaling induced by binding of the chemokine to its receptor leads to the activation of multiple signaling pathways and subsequent release of MMPs into the local environment. These findings may provide a link between chemoattractive mechanisms, growth of tumor cells in bone, and tumor-enhanced bone matrix turnover.